The meiotic protein SWI1 is required for axial element formation and recombination initiation in *Arabidopsis*

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SUMMARY

We report the detailed characterization of SWITCH1 (SWI1) an *Arabidopsis thaliana* protein that has been linked with the establishment of sister chromatid cohesion during meiosis. Using a combination of cytological methods including immunolocalization of meiotic chromosome-associated proteins we show that SWI1 is required for formation of axial elements. Our studies reveal that the *swi1-2* mutation prevents the formation of RAD51 foci during meiotic prophase and suppresses the chromosome fragmentation phenotype of the recombination-defective *dif1-1* mutant. Together, these data suggest that SWI1 may be required for meiotic recombination initiation. Finally we raised an antibody against SWI1 and showed, by immunolocalization coupled with bromodeoxyuridine incorporation experiments, that SWI1 is expressed exclusively in meiotic G_1 and S phase. Thus, SWI1 appears to be required for early meiotic events that are at the crossroad of sister chromatid cohesion, recombination and axial element formation. The possible inter-relationship between these processes and the function of SWI1 are discussed.

Key words: Meiosis, Recombination, Synaptonemal complex, Sister chromatid cohesion.

INTRODUCTION

Meiosis is a central feature in the reproductive program of plants and all other sexually reproducing eukaryotes. In plants such as *Arabidopsis thaliana*, reproduction initiates during the development of the male (anther) and female (ovule) reproductive structures from somatic tissue. During anther development, a group of cells differentiate to form archesporial cells, which in turn give rise to the primary sporogenous cells. These then differentiate into pollen mother cells, the male meiocytes in which meiosis occurs, and primary parietal cells, which give rise to the tapetum, endothecium and middle layer of the anther (Yang et al., 1999). In *A. thaliana* each anther locule contains around 30 meiocytes that enter and proceed through meiosis with a high degree of synchrony to produce tetrads of haploid microspores (Armstrong et al., 2001). These then mature to form pollen grains, the male gametophytes. During female reproductive development in the ovule, an archesporial cell at the top of the ovule primordium differentiates from a single hypodermal cell. This cell then differentiates forming the megaspore mother cell in which meiosis occurs, leading to the formation of four haploid megaspores. The three megaspores closest to the micropyle of the ovule then undergo programmed cell death, whilst the remaining chalazal megaspore develops into the female gametophyte (Yang et al., 1999).

The process of meiosis involves two rounds of chromosome segregation that follow a single round of chromosome duplication leading to the production of haploid gametes. Accurate segregation of chromosomes during meiosis is essential for the long-term survival of individual species, since any error may produce infertility or aneuploid offspring. During meiotic prophase I several crucial events contribute to and determine the subsequent correct partition of genetic material. One of these is the juxtaposition of homologous chromosomes during early prophase I to form bivalents. This process commences at leptotene or in some cases meiotic interphase with the alignment of homologous chromosomes, a phenomenon called pairing (review by Zickler and Kleckner, 1998). During leptotene, each chromosome develops a linear proteinaceous structure called an axial element (AE). In the following zygotene stage, the homologues closely associate via the polymerization of a central element between the two homologous AEs, which are then referred to as lateral elements. This tripartite structure forms the synaptonemal complex (SC). The polymerization of the SC, or synapsis, continues throughout zygotene until pachytene at which stage it is complete. The SC is then disassembled during diplotene (reviewed by Heyting, 1996; Roeder, 1997; Zickler and Kleckner, 1999).

To ensure accurate chromosome segregation at anaphase I

each homologue must remain connected to the other until metaphase I. Since the SC disappears before the end of prophase I, it cannot directly contribute to the links between homologues beyond this point. Beyond pachytene inter-homologue connection is maintained by chiasmata, which are the cytological manifestation of genetic crossover events, in association with sister-chromatid cohesion. Cross-overs are the result of homologous recombination, a process that is initiated at leptotene, by DNA double strand breaks, and is completed by diplotene (Hunter and Kleckner, 2001; Mahadevaiah et al., 2001) (for a review, see Smith and Nicolas, 1998).

The chiasmata are finally released at anaphase I when the sister chromatid cohesion, established earlier in meiotic S phase, is lost along chromosome arms. As a result the homologues are able to segregate to opposite poles of the anaphase I cell. Sister chromatid cohesion is therefore another key element in ensuring accurate chromosome segregation. Release of chromosome arm cohesion is the first in a two-step process, since cohesion continues to be maintained at centromeres, thereby ensuring that the sister chromatids remain associated until metaphase II. At this point centromere cohesion is also lost enabling the second meiotic division to occur at anaphase II. Cohesion is dependent on the activity of the evolutionarily conserved cohesin complex (reviewed by Nasmyth, 2001). Together these processes facilitate chromosome segregation, although many aspects of their interdependency remain unresolved (reviewed by Kleckner, 1996; Nasmyth, 2001; Roeder, 1997; van Heemst and Heyting, 2000; Zickler and Kleckner, 1999).

In plants, relatively few molecular components associated with these meiotic processes have been identified, although there has been recent progress in *A. thaliana* (Bhatt et al., 2001; Caryl et al., 2003; Mercier et al., 2001a). To date, ASY1 is the only known plant protein that is associated with the SC. This protein is required for synapsis and localizes along lateral elements (Armstrong et al., 2002; Caryl et al., 2000; Ross et al., 1997). It shows limited similarity to the yeast meiotic gene *Hop1* (Hollingsworth et al., 1990; Smith and Roeder, 1997), although it has a slightly different spatial and temporal distribution. Five genes required for the catalytic steps of recombination have been described in *Arabidopsis*, namely *RAD50*, *MRE11*, *RAD51*, *DMC1* and *SPO11*. These five genes show strong evolutionary conservation with their counterparts from other species (Bundock and Hooykaas, 2002; Couteau et al., 1999; Doutriaux et al., 1998; Gallego et al., 2001; Grelon et al., 2001). One *Arabidopsis* protein (SYN1/DIF1) has been proposed to have a role in sister chromatid cohesion (Bai et al., 1999; Bhatt et al., 2001). This protein exhibits sequence similarity to the Scc1/Rec8 cohesin family. The observation of meiotic chromosome fragmentation in *syn1/dif1* mutants is also consistent with a functional similarity to Rec8, since the latter is required for double strand break repair during meiotic recombination (Klein et al., 1999). Nevertheless, the role of SYN1/DIF1 in sister chromatid cohesion has not yet been confirmed. Finally, a cyclin-like protein has been shown to be involved in synapsis and recombination (Azumi et al., 2002).

We previously reported the isolation of the *Arabidopsis SWI1* gene [also known as *DYAD* (Agashe et al., 2002)] that is required for completion of meiosis (Mercier et al., 2001b). This gene does not show significant similarity to any known genes, proteins or genomic sequences from other species. The *swi1-*

2 mutation results in a lack of bivalent formation and precocious loss of sister chromatid cohesion during male meiosis. This leads to the presence of 20 chromatids instead of 5 bivalents at metaphase I. The *swi1-2* mutant exhibits the most extreme phenotype from four alleles so far described (Agashe et al., 2002; Cai and Makaroff, 2001; Motamayor et al., 2000), suggesting it is a null allele. This initial study led us to propose that *SWI1* is required for the establishment of sister chromatid cohesion (Mercier et al., 2001b).

We have now carried out a detailed study of SWI1 function using a combination of genetic analysis and immunocytochemistry. Our data clearly establish a pivotal role for SWI1 during early meiosis. We demonstrate by immunolocalization coupled with BrdU incorporation experiments that SWI1 is expressed exclusively in meiotic G_1 and S phase. Examination of *swi1-2* mutant male meiocytes reveals that in addition to synapsis and cohesion defects, axial elements do not assemble, although other axis-associated proteins are present, and recombination is probably not initiated. The central role of SWI1 and the dependence between sister chromatid cohesion, axial element formation, synapsis and recombination are discussed.

MATERIALS AND METHODS

Plant material

Wild-type plants used in this study were *Arabidopsis thaliana* accessions Columbia and Wassilewskija. Mutants were *swi1-2* (Mercier et al., 2001b) and *dif1-1* (Bhatt et al., 1999).

Antibodies

The ASY1 polyclonal antibody used in this study was described by Armstrong et al. (Armstrong et al., 2002). It was used at a dilution of 1:500.

The full-length coding region of RAD51 from *A. thaliana* was cloned into the protein expression vector pET21b (Novagen) as an N terminus fusion to a HIS tag. Upon induction, the HIS-AtRAD51 fusion accumulated as insoluble inclusion bodies in *E. coli* BL21 DE3 (Novagen). Purified, refolded recombinant protein was prepared as described previously (Kakeda et al., 1998) and used to produce a rabbit polyclonal antiserum (ISL, Poole, UK). The working dilution was 1:500.

The SWI1 sequence SPFPVKPLAAKRPLG was synthesized as a multiple antigenic peptide (www.bham.ac.uk/Alta_Bioscience) and used to produce a rabbit polyclonal antiserum (ISL, Poole, UK). The working dilution was 1:400. Immunolocalization in *swi1-2* mutant was used as control.

The SYN1 antibody was raised against a polypeptide derived from amino acid residues 178-353 of the protein (Cai et al., 2003). The working dilution was 1:500.

Cytological procedures

Spreads and immunofluorescence light microscope analyses were performed as described previously (Armstrong et al., 2002). The pre-immune serum was used as a negative control.

The procedure for silver staining chromosome spreads was described by Armstrong et al. (Armstrong et al., 2001). Bromodeoxyuridine (BrdU) pulses were performed and detected using an anti-BrdU kit (Roche) as described previously (Armstrong et al., 2001). Spreads and double immunodetection of BrdU and SWI1 were performed as for other immunolocalization (see above) using the following antibody incubations: rabbit anti-SWI1, 4°C overnight; biotin-conjugated anti-rabbit IgG (Sigma), 37°C for 45 minutes;

mouse anti-BrdU (Roche), 37°C for 30 minutes; Cy3-conjugated streptavidin (Cambio) 37°C for 30 minutes, FITC anti-mouse IgG (Roche), 37°C for 30 minutes, with 3× 5 minutes phosphate-buffered saline washes between each step.

Slides were examined using a Nikon Eclipse T300 microscope. Image capture was achieved using an image analysis system (Smart capture 2, Digital Scientific, UK). Figures were prepared using Adobe PhotoShop 6.0.

Double mutant isolation

Heterozygous *swi1-2* and *dif1-1* mutant plants were crossed and double heterozygotes were identified in the F$_1$ generation. Double homozygotes were then identified in the self-fertilized offspring of these F$_1$ plants by PCR genotyping individual plants using diagnostic primer sets. Homozygous *swi1-2* plants were identified using CAPS markers previously described by Mercier et al. (Mercier et al., 2001b). Plants that were also homozygous for *dif-1* were identified using primers flanking the *dif1-1* Ac element insertion site (5′-TGA-TCTTCGCGTGCAATGTAGC-3′ and 5′-GCCGATGCGAACTTC-AATGG-3′) in combination with an Ac element primer (5′-ATACGATAACGGTCGGTAC-3′), which produce different profiles on +/+, *dif1-1/*+ and *dif1-1/dif1-1* genotypes.

RESULTS

SWI1 is required for axial element formation

In an earlier study we demonstrated that synapsis did not occur in *swi1-2* male meiocytes. This resulted in the transient appearance of ten univalents at prophase I rather than five bivalents found in wild-type meiosis. Subsequently, the sister chromatid cohesion is lost, leading to the presence of 20 chromatids at metaphase I. An important issue that was not resolved because of the DNA staining method previously employed was whether or not the mutant formed an axial element (AE) at leptotene. In order to address this question we have used a silver staining procedure that reveals the protein axes of spread chromosomes (Fig. 1). In wild-type cells at leptotene this revealed the AEs as well organized thin threads (Fig. 1A). An additional feature in meiotic cells that was clearly revealed in this and all other spread preparations of both wild-type and *swi1-2* material was a greatly enlarged nucleolus. During zygotene the thread-like AEs associated pairwise in a progressive manner as the homologues underwent synapsis (Fig. 1B), reaching a maximum at pachytene (Fig. 1C). This resulted in the formation of five bivalents that then condensed at diakinesis (Fig. 1D). In the *swi1-2* mutant, AEs were never observed, the chromosomes appearing thicker and more diffuse. (Fig. 1E). Furthermore the chromosomes underwent progressive condensation without the formation of any of the normal figures of early prophase I (Fig. 1E-H). This resulted in the formation of ten unsynapsed univalent chromosomes (Fig. 1H). Hence, it is clear from these results that the complete lack of synapsis in *swi1-2* male meiocytes is preceded by a failure to form normal AE structures.

In *swi1-2*, ASY1 protein is associated with chromatin but does not organize onto a normal axis.

ASY1 is a meiosis-specific protein that is intimately associated with the chromosome axes during early prophase I (Armstrong et al., 2002). We therefore used this observation as an additional route to investigate AE assembly in male meiocytes of *swi1-2* and wild-type material (Fig. 2). Immunolocalization
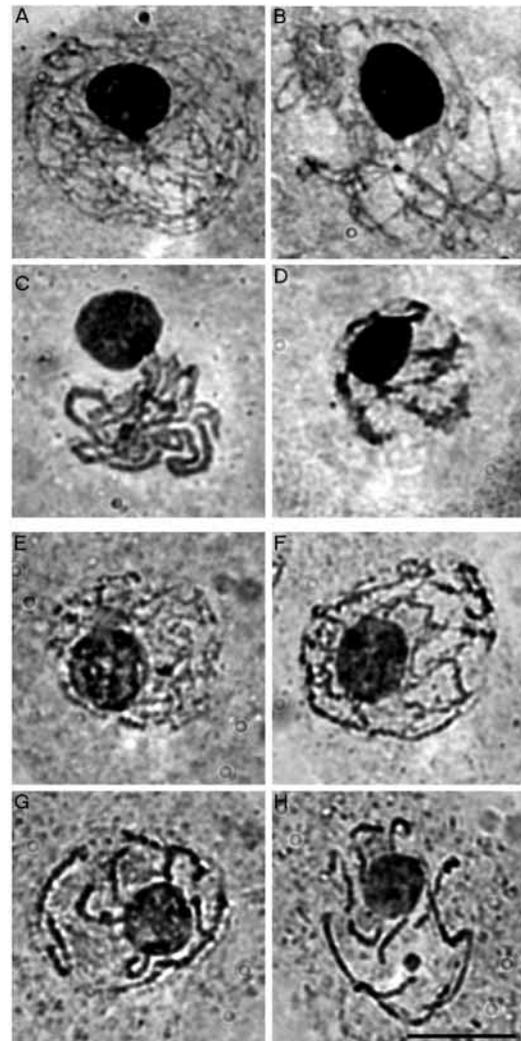
Fig. 1. Silver staining of spread nuclei at meiotic prophase in wild type and *swi1-2* mutant. (A-D) Wild-type meiotic prophase. (A) Leptotene, axial elements appear. (B) Zygotene, synapsis is progressing. (C) Pachytene, synapsis is completed. (D) Diakinesis; five bivalents are condensing. (E-H) *swi1-2* meiotic prophase. Ten univalents progressively condense without any sign of an axial element or synapsis. Scale bar: 10 μm.

of ASY1 in wild-type meiocytes revealed that the protein appeared during G$_2$ as numerous diffuse foci on the chromatin, prior to chromosome condensation (Fig. 2A). At leptotene, ASY1 co-localized with the developing univalent axes, forming thin threads (Fig. 2B) and by pachytene it was associated with the axes of the synapsed chromosomes (Fig. 2C). ASY1 disappeared from chromosomes at diplotene and did not reappear at later stages (data not shown) (see Armstrong et al., 2002). In *swi1-2* interphase nuclei ASY1 localization (Fig. 2D) was indistinguishable from that of wild type. As the ten univalents of *swi1-2* started to condense (Fig. 2E-G), ASY1 co-localized with chromatin (Fig. 2E) and was associated with condensing chromosomes (2F-G). Crucially however, ASY1 did not localize to the threads that typify the leptotene AEs in wild-type meiocytes (compare Fig. 2E,F with B), thereby providing additional strong evidence that *swi1-2* is
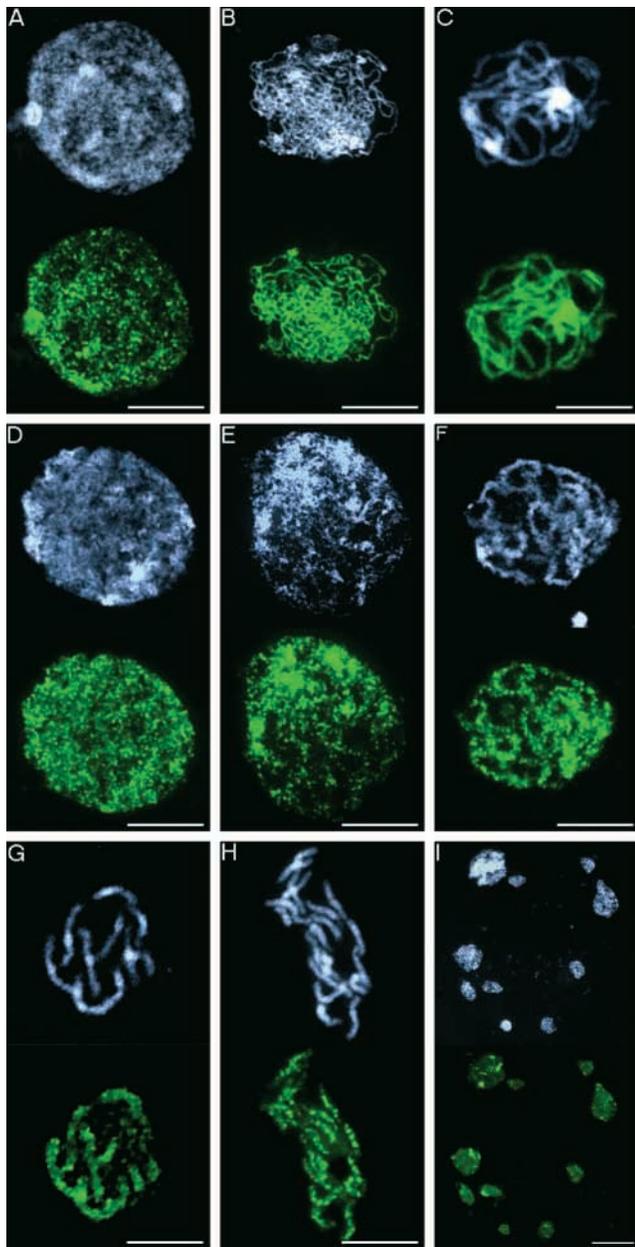
Fig. 2. ASY1 immunolocalization during meiosis in wild type and *swi1-2* mutant. In each panel, the upper picture shows a DAPI-stained nucleus (blue) and the lower one shows the ASY1 immunolocalization (green) on the same nucleus. (A-C) Wild-type meiotic nuclei. (A) Pre-leptotene nucleus, ASY1 is associated with chromatin. (B) Leptotene, ASY1 localization stains the axial elements. (C) Pachytene, ASY1 localizes along synapsed chromosomes. (D-I) *swi1-2* meiotic cells. (D,E) ASY1 localizes on chromatin as it does in wild type. (F,G) ASY1 is present on the condensing ten univalents. (H) The sister chromatid cohesion is released, resulting in 20 free chromatids on which ASY1 is still present. (I) A polyad composed of nine micronuclei on which ASY1 localizes. Scale bars: 10 μm.

unable to assemble normal AEs. Following the loss of sister chromatid cohesion that resulted in the appearance of twenty sister chromatids before the end of prophase I, ASY1 remained

associated with the chromatin (Fig. 2H). Furthermore, this localization was maintained throughout the rest of the aberrant meiosis, such that ASY1 was still present in the micronuclei that arose from random chromosome segregation (Fig. 2I).

The cohesin protein homologue SYN/DIF1 is loaded on to the chromosomes in *swi1-2*

As the *swi1-2* mutant exhibits a sister chromatid cohesion defect, we investigated the behavior of the *Arabidopsis* SYN1/DIF1 protein (hereafter referred to as SYN1), which exhibits homology to the conserved Scc1/Rec8 cohesin sub-unit family. For this purpose, we carried out immunolocalization studies in *swi1-2* and wild-type meiocytes using an antibody raised against recombinant SYN1 protein (Fig. 3). In wild type, SYN1 localized as a diffuse signal on pre-leptotene nuclei (Fig. 3A). At leptotene the SYN1 signal was detected along the developing chromosome axes (Fig. 3B), consistent with its proposed role in sister chromatid cohesion, and remained on the synapsed homologues at pachytene (Fig. 3C). At the end of prophase I the SYN1 signal began to decrease, such that by metaphase I it was barely visible (Fig. 3D), and at later stages was no longer detectable (data not shown) (see also Cai et al., 2003). In the *swi1-2* mutant, the SYN1 distribution at early meiosis was indistinguishable from that of wild-type meiocytes (Fig. 3E). The protein continued to be associated with the ten condensing univalents (Fig. 3F) and following release of cohesion, remained on the twenty chromatids (Fig. 3G). Comparing these stages with wild-type leptotene/pachytene, it can be seen that the fluorescence signal is more diffuse. This most probably reflects a difference in chromosome organization, since *swi1-2* is unable to assemble normal axes. Finally, in contrast to wild-type meiocytes SYN1 remained present up to the end of meiosis (Fig. 3H). From these observations it is clear that the lack of cohesion in the *swi1-2* mutant cannot be attributed to an absence of the SYN1 protein on chromosomes. Interestingly, the localization of SYN1 was remarkably similar to that of ASY1 in both wild type and *swi1-2*.

Formation of RAD51 foci is dependent on SWI1 expression

To study the progression of recombination in *swi1-2* meiocytes we investigated the behavior of the RAD51 protein, which is an essential component of the recombination machinery. RAD51 is involved in both mitotic and meiotic recombination-mediated double strand break (DSB) repair (Masson and West, 2001). We produced and raised an antibody against RAD51 recombinant protein and used this to immunolocalize the protein during meiosis in *swi1-2* and wild-type meiocytes (Fig. 4). During wild-type meiosis, numerous RAD51 foci appeared on leptotene chromosomes, presumably highlighting the sites of recombination initiation (Fig. 4A). Throughout prophase I there was a progressive reduction in the number of signals, such that by early pachytene only a few remained (Fig. 4B) and soon after they also disappeared. In contrast, in *swi1-2,* RAD51 foci were not detectable during meiotic prophase I (Fig. 4C-E), strongly suggesting that recombination does not occur during the aberrant *swi1-2* meiosis. However, unexpectedly, large blob-like RAD51 signals appeared at the end of meiosis in polyad micronuclei (Fig. 4F). This may indicate that RAD51 expression is somehow deregulated in
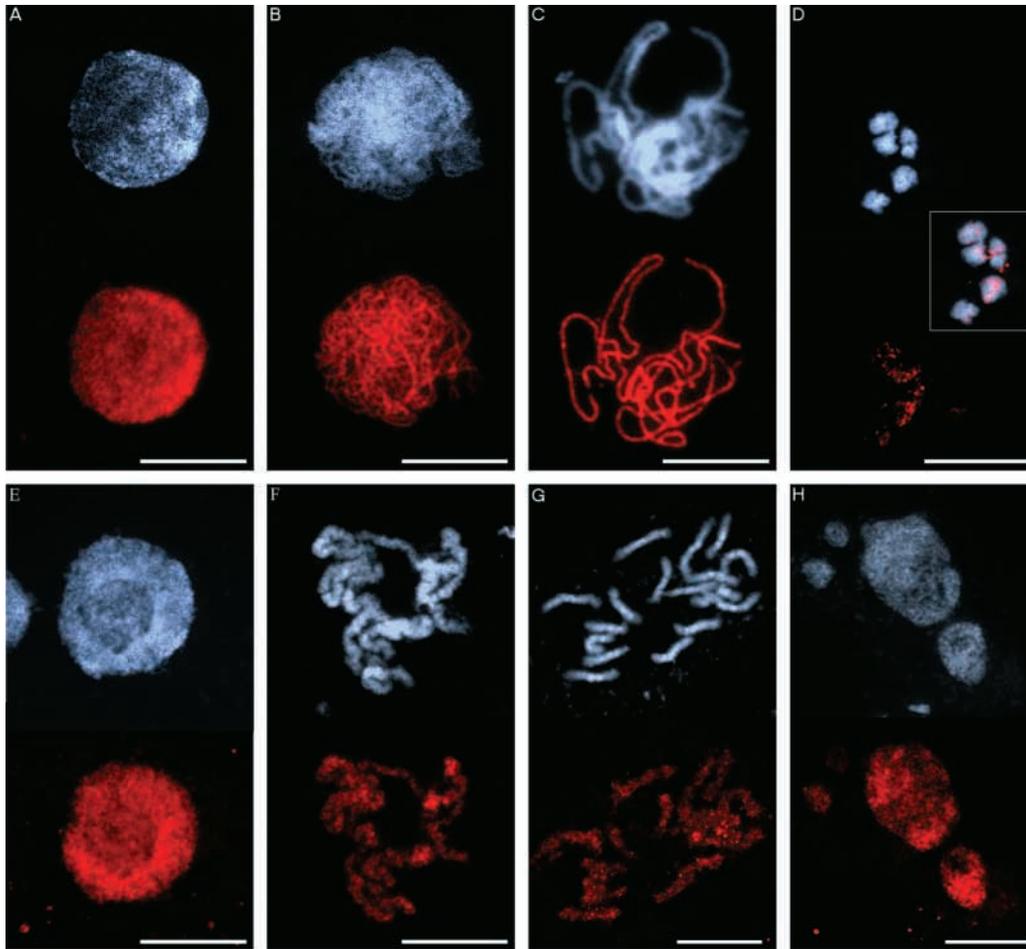
Fig. 3. SYN1/DIF1 immunolocalization during meiosis in wild type and *swi1-2* mutant. In each panel, the upper picture shows a DAPI-stained nucleus (blue) and the lower one shows the SYN1/DIF1 immunolocalization (red) on the same nucleus. (A-D) Wild-type nuclei. (A) Pre-leptotene nucleus, SYN1/DIF1 is associated with chromatin. (B) Leptotene, SYN1/DIF1 localizes along chromosome axes. (C) Pachytene, SYN1/DIF1 immunolocalization stains synapsed chromosomes. (D) Metaphase I, a weak SYN1/DIF1 signal is detected on condensed bivalents. (E-H) *swi1-2* meiotic cells. (E) SYN/DIF1 localizes on chromatin as it does in wild type. (F) SYN1/DIF1 surrounds the 10 condensing univalents. (G) While sister cohesion is released, SYN1/DIF1 remains on the chromatids. (H) A polyad, SYN1/DIF1 localizes in the four nuclei. Scale bars: 10 μm.

these aberrant meiotic products, or perhaps, it is an attempt to repair DNA damage that occurs in these cells as they die.

swi1-2 mutation is epistatic to the *dif1-1* mutant allele of *DIF1/SYN1*

At wild-type meiotic metaphase I, five bivalents were observed (Fig. 5A). In contrast, in the *dif1-1* mutant, a number of chromosome fragments were observed (Fig. 5B) (Bai et al., 1999; Bhatt et al., 1999). In the *swi1-2* mutant, 20 independent chromatids appeared and no sign of fragmentation was observed (Fig. 5C) (Mercier et al., 2001b). In order to study the genetic relationship between them, we produced lines homozygous for both mutations and studied the meiotic behavior of this double mutant (Fig. 5D-F). The observed meiotic defect was very similar to that of the *swi1-2* single mutant, in that sister chromatid cohesion was lost during prophase (Fig. 5D) leading to the presence of 20 condensed chromatids at metaphase I (Fig. 5E). Interestingly, no chromosome fragmentation was observed, showing that *swi1-*

2 suppressed the *dif1-1* fragmentation defect. Nevertheless, the *swi1-2* mutation did not appear completely epistatic to the *dif1-1* mutation as ten condensed univalents were frequently observed in the double mutant (Fig. 5F). Such figures were never observed in the *swi1-2* mutant, where the sister chromatid cohesion is lost before the univalents condense. This result suggests that in the absence of DIF1 (SYN1) chromosome condensation occurs in a *swi1-2* genetic background.

SWI1 is expressed in meiotic G_1 and S phase

SWI1 localization has been previously studied using a SWI1::GFP fusion construct (Mercier et al., 2001b). These studies revealed that the GFP signal appeared exclusively in meiotic interphase nuclei, and disappeared before leptotene. To confirm this expression pattern we carried out immunolocalization of SWI1 using an antibody raised against a synthetic peptide based on residues SPFPVKPLAAKRPLG of SWI1 (Fig. 6). This confirmed that the protein was present
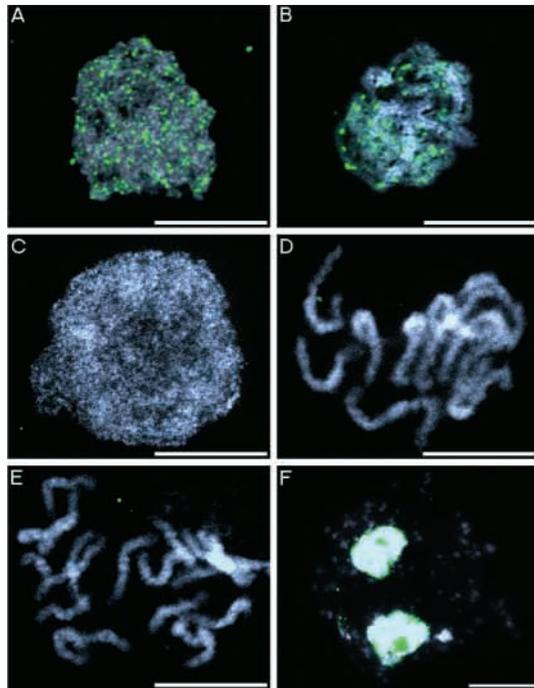
Fig. 4. RAD51 immunolocalization during meiosis in wild type and *swi1-2* mutant. RAD51 appears in green and the DAPI-stained DNA in blue. (A) Wild-type leptotene, RAD51 immunolocalization reveals numerous spots on chromosomes. (B) Wild-type pachytene, the number of RAD51 spots has decreased. (C-F) *swi1-2* meiotic cells. (C) Early meiotic cells, no RAD51 signal. (D) 10 condensing univalents, no RAD51 signal. (E) 20 free chromatids, no RAD51 signal. (F) A polyad, RAD51 appears in the two nuclei. Scale bars: 10 μm.

Fig. 5. s*wi1-2* mutation suppresses the *dif1-1* chromosome fragmentation phenotype. (A) Metaphase I in a wild-type male meiocyte. Five bivalents are observed. (B) *dif1-1* meiocyte exhibiting chromosome fragmentation. (C) *swi1-2* meiocyte at the end of prophase. Twenty chromatids are observed. (D,E) *swi1-2/dif1-1* double mutant meiocytes. (D) Sister chromatid cohesion is lost at the end of prophase I. (E) The resulting 20 free chromatids at metaphase I. (F) Some cells contained 10 condensed univalents. Scale bar: 10 μm.

in nuclei at meiotic interphase (Fig. 6A). By leptotene the SWI1 signal was no longer detectable (Fig. 6B) and was not found in subsequent meiotic stages (data not shown). These results are entirely consistent with the earlier SWI1::GFP studies, save one apparent difference. It is quite clear from the immunolocalization studies that SWI1 is present in the nucleolus and may to some extent accumulate there (Fig. 6A,D). In contrast, confocal imaging suggested that SWI1::GFP was excluded from this structure, presumably because of the increased molecular mass of the fusion protein preventing translocation into the nucleolus. It is possible that the nucleolus functions as a reservoir and somehow regulates protein function/availability (Visintin and Amon, 2000). But, even if SWI1 is subject to such regulation, it cannot be essential, since the SWI1::GFP fusion is apparently excluded from the nucleolus, yet is able to restore the fertility of *swi1* mutants (Mercier et al., 2001b) (data not shown).

Whilst the immunolocalization and GFP fusion data concur in showing that SWI1 is expressed exclusively at pre-leptotene interphase, their precision is insufficient to determine if this is in G_1, S or G_2 phase. To address this problem we carried out SWI1 immunolocalization in conjunction with BrdU incorporation experiments. BrdU is a thymidine analogue that can be incorporated in DNA during replication. S phase meiotic nuclei may be identified using immunocytology on samples taken following a 2-hour pulse with BrdU. Subsequent

stages through the meiotic sequence may be obtained by taking further samples at 2-hour intervals (Armstrong et al., 2001). In a first experiment, samples were collected immediately after the 2-hour pulse. The BrdU-positive cells were then identified as being in S or very early G_2 phase (Diagram 1-Fig. 6C). These cells were found to be expressing SWI1 (Fig. 6D, 11 cells out of 11), showing that the protein is present throughout the whole meiotic S phase. In the same experiment, a proportion of the cells expressing SWI1 were not BrdU labeled (Fig. 6E), indicating that SWI1 expression is not restricted to S phase. In a second experiment, we delayed sample collection for 2 hours, 6 hours or 10 hours after the end of the pulse (returning to BrdU-free medium during these times), such that an increasing proportion of the BrdU-labeled cells would have progressed into G_2 (Diagram 2-Fig. 6C). After 2 hours, only half of the BrdU-positive meiotic cells were expressing SWI1 (Fig. 6F, 6 cells out of 12). After 6 hours and 10 hours the BrdU-positive meiotic cells were no longer expressing SWI1
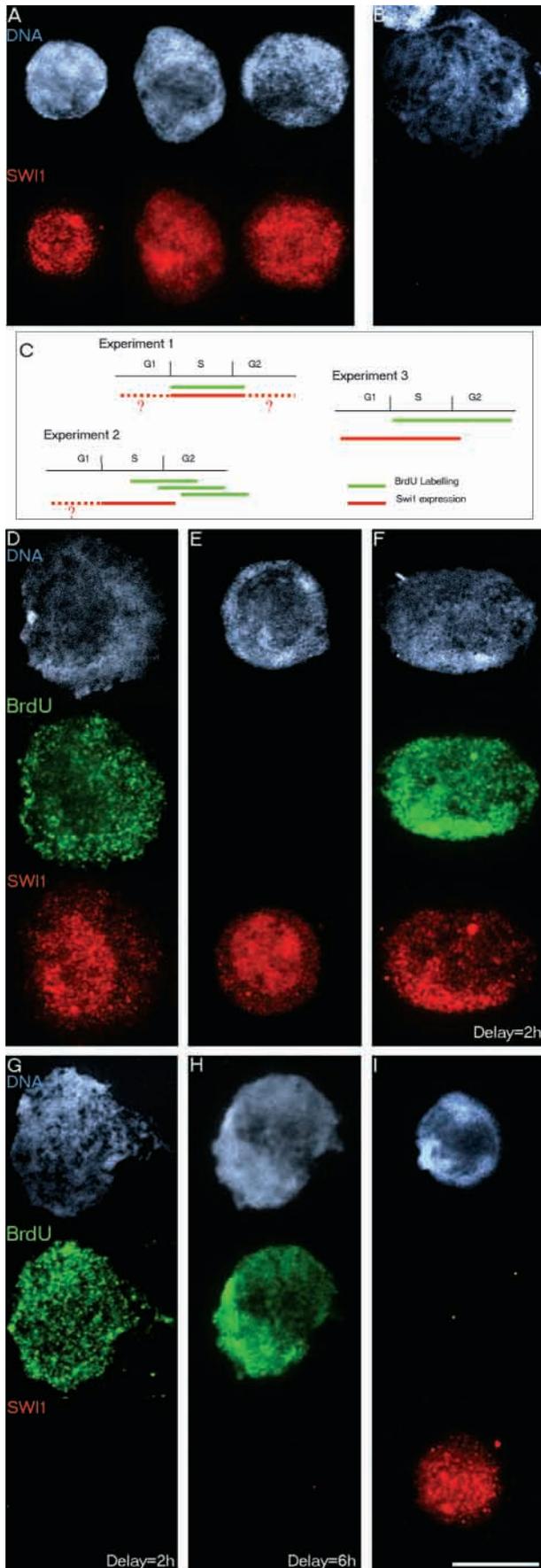
Fig. 6. SWI1 immunolocalization. (A) Three examples of meiotic cells expressing SWI1. (B) SWI1 is not detected at leptotene stage. (C) Diagrams outlining the three kinds of BrdU incorporation experiment. (D-I) Double localization of SWI1 and incorporated BrdU. In each panel, the upper picture shows a DAPI-stained nucleus (blue), the middle one the BrdU immunolocalization (green), and the lower one shows the SWI1 immunolocalization (red) on the same nucleus. (D) Experiment 1. Tissues were harvested directly at the end of the pulse. The cell shown is at S phase, according to the BrdU labeling, and expresses SWI1. (E-H) Experiment 2. Samples were harvested 2 hours, 6 hours or 10 hours after the end of the 2-hour pulse. After a 2-hour delay, half of the BrdU-positive cells express SWI1 (E) while the other half do not (F). After a 6-hour or a 10-hour delay, BrdU-positive cells do not express SWI1 (G-H). (I) Experiment 3. Samples were harvested at the end of an 8-hour BrdU treatment. Small meiotic cells express SWI1, and are BrdU negative, indicating they are in G_1. Scale bar: 10 μm.

(Fig. 6G; 0 cells out of 17 and 7, respectively). These results taken in conjunction with the result of the first experiment suggest that SWI1 is not present during the G_2 stage. In a third experiment, we performed a continuous BrdU application for a period of 8h before sampling. This treatment ensures that all cells in S and most G_2 cells would be BrdU positive (Diagram 3-Fig. 6C). This revealed a population of small cells that were expressing SWI1, but were not labeled by the BrdU immunolocalization (Fig. 6I). This result strongly suggests that SWI1 is present in G_1 cells prior to the initiation of DNA replication. Together, these experiments indicate that SWI1 is expressed at meiotic G_1 and S phase, but not during G_2 or later stages of meiosis.

DISCUSSION

SWITCH1 is involved in sister chromatid cohesion, axial element formation and recombination

A previous study led us to propose that SWI1 is involved in the establishment of sister chromatid cohesion during male meiosis (Mercier et al., 2001b). We have conducted a detailed study of SWI1 function. Light microscope observation of silver-stained spread meiotic cells revealed that normal AE do not form in the *swi1-2* mutant. Further evidence substantiating this conclusion was obtained by investigating the distribution of the ASY1 and SYN1/DIF1 proteins in *swi1-2*. They are loaded on to the chromatin as normal, but fail to organize into the thread-like structures found in wild-type cells. Another striking feature revealed by the immunolocalization studies was that both proteins remained associated with chromosomes throughout meiosis. Thus although these proteins are expressed normally and loaded onto the chromatin, the failure in AE formation affects their ability to organize correctly during early prophase I and this in turn has knock-on consequences for their subsequent processing and removal from the chromosomes.

The lack of chiasmata between the univalents that are transiently present in *swi1-2* (Mercier et al., 2001b) (see also Fig. 1H, Fig. 2G, Fig. 4D) implies that genetic crossing-over (CO) does not occur in the *swi1-2* mutant. Together with the lack of any detectable chromosome fragmentation this suggests that DSBs that initiate recombination are not produced in the mutant. Our analysis of an *Arabidopsis* line homozygous for

both *swi1-2* and *dif1-1* mutations concurs with this hypothesis. The *dif1-1* mutation results in extensive chromosome fragmentation during meiosis (Bhatt et al., 1999) (see also Fig. 5A). The fragmentation phenotype is suppressed by a *spo11* mutation, indicating that the *dif1-1* phenotype is due to a failure in the repair of SPO11-mediated DSBs (Anuj Bhatt and Mathilde Grelon, personal communication). We showed that the *swi1-2* mutation also suppressed the *dif1-1* fragmentation phenotype, further indicating that *swi1-2* mutation impairs recombination initiation. Nevertheless, it is also possible that in a *swi1-2* background DSBs are produced and are repaired via some alternative pathway such as gene conversion during a transient interaction between the homologues or recombination between the sister chromatids prior to their separation. However, our analysis of the behavior of RAD51 in the *swi1-2* mutant provides evidence against these alternative possibilities. Rad51 is an evolutionarily conserved protein essential for DSB repair by homologous recombination regardless of whether the template is the sister chromatid or the homologous chromosome (review by Masson and West, 2001). In contrast to wild-type meiocytes, no RAD51 foci were detected on *swi1-2* chromosomes during meiotic prophase. This finding is consistent with a failure of *swi1-2* to initiate recombination.

On the basis these experiments it seems that SWI1 has a pivotal role in meiotic chromosome function, as it appears to be required for sister chromatid cohesion, axial element formation and recombination.

How are the functions of SWI1 in sister cohesion, axial element formation and recombination inter-related?

In this study we have shown that SWI1 is expressed at an early stage in meiosis. It is first detectable in G_1, remains present in S phase and disappears probably as soon as S phase finishes. On the basis of this expression pattern we propose that the primary defect of *swi1-2* is a lack of cohesion initiation [already suggested by Mercier et al. (Mercier et al., 2001b)]. Considerable data, mainly from yeast, suggest a mechanism whereby cohesion is established during mitotic or meiotic S phase, when the two sister chromatids originate (reviewed by Nasmyth, 2001). For example, a delay in expression of the cohesin sub-unit Scc1 after the mitotic S phase leads to a lack of cohesion in budding yeast (Uhlmann and Nasmyth, 1998). *Schizosaccharomyces pombe* cells that undergo a meiosis after having performed a mitotic S-phase have the same phenotype as the *rec8* mutant, showing that the meiosis-specific cohesion system is built as early as S-phase (Watanabe et al., 2001). Some proteins, called adherins, have been isolated, that are required for the loading of cohesins at G_1 stage (Ciosk et al., 2000; Furuya et al., 1998) while others directly link replication and cohesion establishment (review by Carson and Christman, 2001). These proteins have a mitotic role and are thought to have a similar meiotic function, although thus far, this has been formally demonstrated only for the *Coprinus cinereus* adherin Rad9 (Cummings et al., 2002). At present, SWI1 is the only protein to be described that is involved specifically in meiotic sister chromatid cohesion establishment. Moreover, detection of the SWI1 protein prior to meiotic S phase places it as the earliest acting meiotic protein described to date in plants. Hence, it seems possible that it may prove to be a key target

for the as yet unknown factors that specify this developmental pathway.

The *swi1-2* defect in axial element formation and as a consequence the lack of SC, is probably a secondary defect of the sister chromatid cohesion defect, particularly as SWI1 is no longer detectable by the time the AE begins to form in leptotene. In a variety of species, AE formation is dependent on the previous establishment of sister chromatid cohesion (reviewed by van Heemst and Heyting, 2000): the cohesin mutants *rec8* and *smc3* from *Saccharomyces cerevisiae*, and *rec8* from *Caenorhabditis elegans* do not form AEs (Klein et al., 1999; Pasierbek et al., 2001). The *Sordaria macrospora spo76-1* mutant also exhibits a meiotic cohesion defect that results in abnormal and partially split AEs (van Heemst et al., 1999). Similarly, the fission yeast *rec8* mutant does not form a linear element, an AE-like structure (Molnar et al., 1995). All the cohesin sub-units studied so far in various species localize along the axial cores in leptotene (review by Nasmyth, 2001). Furthermore in mammals AE-like structures containing cohesin sub-units are found even in the absence of the AE core constituent SCP3 (Pelttari et al., 2001). Finally, some mammalian cohesin sub-units interact with AE components (Eijpe et al., 2000). Taken together, these data add to a growing consensus that cohesin axes provide the base for AE formation. Our characterization of the *swi1-2* phenotype accords well with such a model.

SWI1 seems to be required for meiotic recombination initiation (see above). Several possible explanations might account for this observation. First, the recombination initiation defect may be a consequence of the lack of AEs. However, the yeast *smc3* and *Rec8*, and apparently the *C. elegans Rec8* mutants form DSBs in the complete absence of AE (Klein et al., 1999; Pasierbek et al., 2001) suggesting that the latter is not required for recombination initiation. Nevertheless, we cannot exclude the possibility that it is not the case in *Arabidopsis* since, so far, no *Arabidopsis* mutants other *than swi1-2* have been described that exhibit a lack of AE. Another possibility is that the lack of DSB formation in *swi1-2* is due to the absence of sister chromatid cohesion. However, all the cohesion mutants described to date are able to initiate recombination. In the *S. cerevisiae rec8* and *smc3* cohesion mutants, Spo11-mediated DSBs occur but are not repaired (Klein et al., 1999). Similarly, the *C. elegans* meiocytes lacking Rec8 and the *A. thaliana syn1/dif1* mutants exhibit chromosome fragmentation (Pasierbek et al., 2001; Bhatt et al., 1999; Bai et al., 1999). In the *Sordaria spo76-1* mutant, a limited reduction in the number of Rad51/Dmc1 foci suggests that recombination initiation is nearly normal (van Heemst et al., 1999). If it is the case that DSB formation is independent of the sister chromatid cohesion establishment, then it could suggest a role for SWI1 in recombination initiation that is distinct from its cohesion function. Several lines of evidence indicate a mechanistic link between recombination initiation and DNA replication (Borde et al., 2000; Cha et al., 2000; Keeney, 2001). Some authors proposed that passage of the replication fork is required to establish a structure permissive for DSB formation, or/and that factors involved in DSB formation, such as Spo11, might assemble onto DNA during replication. One can hypothesize that SWI1 may be involved in such an S-phase process, in addition and contemporaneous to its function in sister chromatid cohesion establishment.

Functional considerations

SWI1 thus appears required for sister chromatid cohesion establishment, consequently for AE and SC formation, and, possibly independently, for recombination initiation. Nevertheless, the actual function of SWI1 remains a matter of speculation. We have previously proposed (Mercier et al., 2001b) that SWI1 could perform a similar function to that of the yeast proteins Scc2/Mis4 and Scc4, which permit the association of the cohesin complex with chromatin (Ciosk et al., 2000; Furuya et al., 1998). However this hypothesis appears to be ruled out by our finding that the Rec8 homologue SYN1/DIF1 localizes on *swi1-2* chromosomes (Fig. 3). At this stage, we cannot exclude the possibility that SWI1 establishes cohesion in a manner analogous to the yeast Eco1/Ctf7 or Eso1 protein (Skibbens et al., 1999; Tanaka et al., 2000; Toth et al., 1999), by interacting with the cohesin protein after the latter has loaded onto the chromosomes. Alternatively SWI1 could have a role in cohesion (and possibly recombination) that is completely independent of SYN1/DIF1, *via* loading or modifying other components during G_1 or S phase. However, such candidates remain to be identified.

Finally, this study highlights an interesting aspect of developmental regulation of meiosis in *Arabidopsis* that remains to be resolved. Several genes including SWI1, ASY1 and SYN1 encode proteins that are on current evidence entirely specific to meiosis, yet transcripts from them are detected in other vegetative tissues. This suggests that regulation at the level of translation may be a significant feature of meiosis in *Arabidopsis*.

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